

Comparison of direct and indirect fluorescent antibody methods for staining *Treponema pallidum*

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Both direct and indirect fluorescent antibody methods have been used to examine body fluids for the presence of *Treponema pallidum*. In the direct method (Yobs, Brown, and Hunter, 1964), fluorescein isothiocyanate (FITC)-labelled anti-*T. pallidum* globulin is layered directly upon the specimen suspected of containing treponemes. The indirect method is a double-layer procedure in which the specimen to be examined is first coated with syphilitic serum containing unlabelled anti-*T. pallidum* globulin, and this, in turn, is layered with FITC-labelled globulin directed against the species from which the serum was derived (Edwards, 1962). We have encountered no studies in which data comparing the two methods were presented. Because some investigators have expressed a preference for one method or the other (Israel, 1969; Wells and Smith, 1967; Rice, Dunlop, Jones, Hare, King, Rodin, Mushin, and Wilkinson, 1970), we wished to compare both methods concurrently, under similar conditions, to determine whether, with current reagents, one offered significant advantages. This information might be helpful in deciding upon a standard method to be used by all investigators searching for spiral organisms in body fluids.

Material and methods

ANTIGEN SLIDES

T. pallidum, Nichols strain, used as antigen for the fluorescent treponemal antibody-absorption test (FTA-ABS), was prepared by re-suspending the lyophilized organisms in sterile distilled water (U.S. Department of Health, Education, and Welfare, 1969). Reiter treponemes were taken from 8-day cultures. Those organisms designated as 'washed' were washed three times in saline and packed by centrifugation in an attempt to remove rabbit protein adsorbed to their surface. Fresh pathogenic *T. pallidum*, Nichols strain, and *T. cuniculi* were harvested from rabbit testicles, inoculated 2 to 4 weeks previously. *T. carateum* was obtained from fluid expressed from skin lesions in inoculated chimpanzees (Kuhn, Medina, Cohen, and Vegas, 1970). *T. pertenue* was obtained from skin lesions developing in macaques inoculated with a

Venezuelan strain of this organism provided by Dr. Raphael Medina. Previously this strain had been cultivated in Syrian hamsters. Fresh *T. microdentium* was obtained from mouth washings.

All specimens were placed within pre-etched circles on alcohol-cleaned glass slides and allowed to dry in air. The slides were then fixed in acetone at room temperature for 10 min., air dried, and stored at -20°C. (U.S. Department of Health, Education, and Welfare, 1969).

STAINING TECHNIQUE

The direct staining technique was performed by layering FITC-labelled anti-*T. pallidum* globulin of rabbit origin, previously absorbed with whole Reiter treponemes, VDRL lot 169 (Mothershed and Bullard, 1968), on to the antigen slides. The conjugate had been diluted 1:40 either in phosphate buffered saline (PBS) pH 7.2 (U.S. Department of Health, Education, and Welfare, 1969), containing 2 per cent. Tween 80, or in 0.5 M carbonate buffer pH 9.0 (Cherry, Goldman, and Carski, 1960), containing 2 per cent. Tween 80. The conjugate was allowed to react at room temperature for 30 min. in a moist chamber. After staining, the slides were rinsed twice either in PBS pH 7.2, or in 0.5 M carbonate buffer pH 9.0 for 5 min. and then in distilled water for 5 min. The slides were then blotted dry with bibulous paper and a small drop of mounting medium was applied. The mounting medium was prepared from nine parts glycerin to one part buffer. The buffers used for mounting were: PBS pH 7.2; 0.01 M phosphate buffer pH 7.2; 0.01 M carbonate buffer pH 9.0; 0.5 M carbonate buffer pH 9.0. Coverslips were then affixed.

In the indirect technique, the antigen slides were layered with 4+ reactive human syphilitic control serum, VDRL lot 691, previously diluted with four parts VDRL FTA-ABS test sorbent, lot 694. This was allowed to react in a moist chamber for 30 min. at 37°C. The slides were then washed twice either in PBS pH 7.2, or in 0.5 M carbonate buffer pH 9.0, for 5 min. followed by one rinse in distilled water for 5 min. The slides were blotted dry and overlaid with FITC-labelled anti-human globulin (VDRL lot 69169C), rabbit origin. The conjugate had been diluted 1:100 either in PBS pH 7.2, containing 2 per cent. Tween 80 or in 0.5 M carbonate buffer pH 9.0, containing 2 per cent. Tween 80. After a second 30-min. incubation in a moist chamber at 37°C., the slides were washed as before in one of the two buffers and in

distilled water. They were blotted dry and mounted in the various glycerin-buffer mixtures described for the direct technique, and coverslips were affixed.

MICROSCOPIC READING

Observations were made at 540 \times magnification with a Leitz ultraviolet SM microscope, using a dark-field condenser and an HBO 200 mercury lamp. A BG-12 3mm. thick exciting filter and OG-1 barrier filter were used. All specimens were read in a blind fashion by one observer, and graded as bright fluorescence, moderate fluorescence, weak fluorescence, or no fluorescence. All slides were prepared and read on three or four separate occasions.

Results

Table I summarizes the results obtained when lyophilized reconstituted *T. pallidum* (FTA-ABS test antigen), fresh *T. pallidum* Nichols strain, and washed and unwashed Reiter treponemes were stained by both the direct and indirect techniques, one of the two buffer systems being used throughout.

Both the FTA-ABS antigen and fresh Nichols strain *T. pallidum* stained brightly with the direct and indirect techniques when the PBS buffer system was used. The pH 9.0 carbonate buffer severely quenched the fluorescence of these organisms. The unwashed Reiter organisms stained weakly with the indirect method using PBS. This fluorescence was abolished when the organisms had been washed in saline. Varying the buffer used in the mounting media (0.01 M phosphate buffer pH 7.2; PBS pH 7.2; 0.01 M carbonate buffer pH 9.0; 0.5 M carbonate buffer pH 9.0) caused no change in the fluorescence of the organisms.

Since Israel (1969) has used the 0.5 M carbonate buffer pH 9.0 in the indirect technique successfully, we decided to evaluate the action of the buffer more fully in our system. All combinations of the carbonate buffer and PBS were tested in the three steps

of the indirect test as shown in Table II. Bright fluorescence was obtained when PBS was used throughout the technique or when the carbonate buffer was used only to wash the excess serum-sorbent mixture from the antigen slides. Fluorescence was reduced severely when the carbonate buffer was used throughout. Moderate fluorescence remained with other combinations of the buffers.

TABLE II *Action of 0.5 M carbonate buffer, pH 9.0, and PBS, pH 7.2, upon indirect fluorescent antibody method using FTA-ABS test antigen*

First wash (to remove serum and sorbent)	Diluent for fluorescent conjugate	Second wash (to remove conjugate)	Fluorescence
PBS	PBS	PBS	Bright
Carbonate	Carbonate	Carbonate	Weak
PBS	Carbonate	Carbonate	Moderate
PBS	PBS	Carbonate	Moderate
Carbonate	Carbonate	PBS	Moderate
Carbonate	PBS	PBS	Bright
PBS	Carbonate	PBS	Moderate
Carbonate	PBS	Carbonate	Moderate

The indirect and direct methods, using the PBS system, were then applied to a group of pathogenic treponemes, fresh *T. microdentium*, and washed Reiter treponemes (Table III). All pathogenic treponemes stained equally well with both techniques. Neither method stained fresh *T. microdentium* or washed Reiter treponemes.

Discussion

Various modifications of direct and indirect techniques have been used to detect treponemes in body fluids. Several workers originally used a direct staining technique with diluent buffered washes, and mounting media containing PBS pH 7.2 (Smith and Israel, 1968; Wells and Smith, 1967; Wilkinson, 1968). Goldman and Girard (1967, 1968) have used

TABLE I *Comparison of direct and indirect fluorescent antibody techniques with PBS, pH 7.2, or 0.5 M carbonate, pH 9.0, buffer systems*

Organism	Direct method		Indirect method	
	PBS pH 7.2	Carbonate pH 9.0	PBS pH 7.2	Carbonate pH 9.0
Lyophilized, reconstituted <i>T. pallidum</i> , Nichols strain (FTA-ABS test antigen)	Bright*	Weak	Bright	Weak
Fresh <i>T. pallidum</i> , Nichols strain	Bright	Weak	Bright	Weak
Cultivated Reiter treponemes				
Unwashed	Negative	Negative	Weak	Negative
Washed	Negative	Negative	Negative	Negative

*Fluorescent staining was reported as: Bright, corresponding to 3-4+ in a 1+ to 4+ scale
Moderate, corresponding to 2+

Weak, corresponding to 1+
Negative

TABLE III Comparison of direct and indirect fluorescent antibody methods in staining of various organisms
PBS pH 7.2 was used throughout

Organism	Intensity of fluorescence	
	Direct method	Indirect method
Lyophilized, reconstituted <i>T. pallidum</i> , Nichols strain (FTA-ABS test antigen)	Bright	Bright
Fresh <i>T. pallidum</i> , Nichols strain	Bright	Bright
Fresh <i>T. cuniculi</i>	Bright	Bright
Fresh <i>T. pertenue</i>	Bright	Bright
Fresh <i>T. carateum</i>	Bright	Bright
Fresh <i>T. microdentium</i>	Negative	Negative
Cultivated Reiter treponemes, washed	Negative	Negative

both direct and indirect methods, while Golden employed only the latter (Golden, Watzke, Lindell, and McKee, 1968). More recently, some workers (Israel, 1969; Rice, Dunlop, Jones, Hare, King, Rodin, Mushin, and Wilkinson, 1970) have begun using the indirect method exclusively, and Israel (1969) has suggested using 0.5 M carbonate buffer pH 9.0. Reasons for preferring the indirect technique have included claims of increased intensity of fluorescence and ease of photographing the fluorescent organisms.

In the present study, both direct and indirect methods stained pathogenic treponemes equally well when the PBS system pH 7.2 was used. Good quality photographs of the fluorescing organisms were made with both black and white (ASA 400) and colour (ASA 500) film, using exposure times of 2 to 3 min. Varying the pH and molarity of the buffer used in the glycerin-mounting medium had no effect on the observed fluorescence.

When both the direct and indirect techniques were used with the 0.5 M carbonate buffer system pH 9.0, the fluorescence of pathogenic treponemes was severely diminished. Further evaluation showed that this effect could be traced to a direct action of the buffer primarily upon the FITC-globulin conjugate but also upon the *T. pallidum*-human syphilitic serum complex. Staining was consistently diminished to a moderate level when the buffer was used to dilute the FITC conjugate or to wash the excess conjugate from the slide. Since fluorescence remained bright when the carbonate buffer was used only to wash the excess human serum from the antigen slide, one might infer that the alkaline buffer quenched fluorescence primarily by causing dissociation of

the FITC-antibody complex as has been suggested by M. Goldman (1968). However, the greatest diminution of fluorescence occurred when the buffer was used in all steps of the indirect technique, indicating that the buffer also exerts some deleterious effect upon the *T. pallidum*-human serum complex.

Since both direct and indirect fluorescent antibody techniques performed equally well in terms of treponeme morphology and brightness of fluorescence, no one method can be recommended by these criteria alone. However, when specificity for pathogenic treponemes is considered, some reservation must be expressed with the indirect method. Although the present experiment, using one syphilitic serum, and the work of J. N. Goldman (1968) demonstrated good specificity for pathogenic treponemes with the indirect method, other investigators (Wilkinson and Ferguson, 1968) have shown that, when strongly reactive human syphilitic serum is used in the indirect technique, sorbent may not remove all group anti-treponemal antibodies, and non-pathogenic treponemes, such as Reiter, may fluoresce. (In the present experiment, the weak fluorescence of unwashed Reiter treponemes, seen with the indirect technique, was probably caused by non-specific binding of human syphilitic serum to the rabbit serum in which the Reiter treponeme had been cultivated. The fluorescence disappeared when the organisms had been washed in saline before application to the glass slides.) Therefore, if the indirect technique is to be used, the investigator must either first evaluate the specificity of the sorbed syphilitic serum for pathogenic organisms, or examine fluorescing spiral forms for morphology and brightness alone, without regard for suspected pathogenicity.

Summary

In a comparative 'blind' study, both direct and indirect fluorescent antibody techniques stained *T. pallidum* and other pathogenic treponemes equally well. Severe decrease in fluorescence occurred when either method was used with a pH 9.0 buffer system, as compared to good results obtained when pH 7.2 buffer was used. Although neither method stained selected non-pathogenic treponemes, reservations are expressed when the indirect method is used without an evaluation of the specificity of the sorbed syphilitic serum.

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Comparaison des méthodes directe et indirecte de marquage à l'anticorps fluorescent pour *Treponema pallidum*

SOMMAIRE

Dans une étude comparative aveugle, les méthodes directe et indirecte utilisant l'anticorps fluorescent pour le marquage de *T. pallidum* et des autres tréponèmes pathogènes donnèrent les mêmes bons résultats. Une baisse marquée de la fluorescence survint, avec les deux méthodes, lorsqu'un système tampon à pH 9 fut utilisé, alors que, par comparaison, de bons résultats furent obtenus avec un pH de 7,2. Quoiqu'aucune des méthodes ne put colorer des tréponèmes non pathogènes choisis, on fait des réserves sur la méthode indirecte si elle est utilisée sans avoir établi la spécificité de l'absorption utilisée pour le sérum syphilitique.